Exhibit

Endothelial-Dependent Procoagulant and Anticoagulant Mechanisms

Recent Advances in Understanding

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Modulation of endothelial cell coagulant function is one of a group of changes common to many cytokine-mediated events. Changes that 1) cause migration of leukocytes, 2) increase vascular permeability, and 3) increase the thrombotic potential occur at atherosclerotic arterial branch points, in tumor vasculature, and at sites of inflammation. Regulation of procoagulant activity on the luminal surface of the vessel is crucial and is achieved by presentation of a predominantly anticoagulant surface on the endothelium. Inflammatory mediators can cause a decrease in the expression of the anticoagulant mechanisms and up-regulation of the procoagulant tissue factor. However, under these conditions very little tissue factor is exposed to the blood; instead it is sequestered under the endothelium and presumably becomes exposed only when significant vascular damage is present. Inhibition of intravascular coagulation by factor IXai without impairment of extravascular hemostasis suggests that when tissue factor concentrations are low, the continued generation of factor Xa is dependent on the presence of factor IXa. The demonstration that the blockade of factor IXa is selective for prevention of intravascular thrombus formation suggests a new means for managing intravascular thrombosis without altering the normal hemostatic mechanisms. ***(Texas Heart Institute Journal 1994;21:86-90)***

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The endothelium plays an important role in hemostasis, inflammation, and atherosclerosis. It has become increasingly evident that the functional phenotype of endothelial cells is regulated by inflammatory cytokines, such as interleukin 1 (IL 1) and tumor necrosis factor (TNF). The development of atherosclerotic plaque is dependent on the attraction and binding of leukocytes to the endothelium, their migration through the endothelial monolayer, and subsequent formation of fatty streaks and thrombi.[1] This process is probably facilitated by inflammatory cytokines, such as TNF, IL 1, and vascular permeability factor/vascular endothelial growth factor (VPF/VEGF). These act on the endothelium to promote and regulate the movement of cells and solutes into tissues 1) by producing and binding chemotactic cytokines (e.g., IL 1 and IL 8), 2) by increasing the expression of leukocyte adhesion molecules (e.g., selectins), 3) by altering vascular permeability, and 4) by modulating coagulant mechanisms.[1] This review focuses mainly on the capability of the endothelium to modulate coagulant function and on regulation of the endothelium by cytokines.

Considering the adverse consequences of thrombosis, it is not surprising that a number of complex interlocking pathways are devoted to controlling coagulation. The coagulant phenotype of the endothelial cell surface can be seen as a balance between 2 opposing pathways—namely, procoagulant and anticoagulant mechanisms—and activation or deactivation of any of its components can potentially tip the balance. Quiescent endothelium normally maintains an anticoagulant surface (Fig. 1). In vitro and in vivo studies have shown several conditions under which this balance can be tipped toward the procoagulant pathways via an autocrine loop: through production of IL 1, through leukocyte-derived cytokines such as TNF, or by locally produced factors such as VPF/VEGF.[2,3]

Coagulation Cascade

The pro- and anticoagulant enzymes circulate in the blood as inactive zymogens, which are activated by a cascade of enzyme-substance interactions of the intrinsic

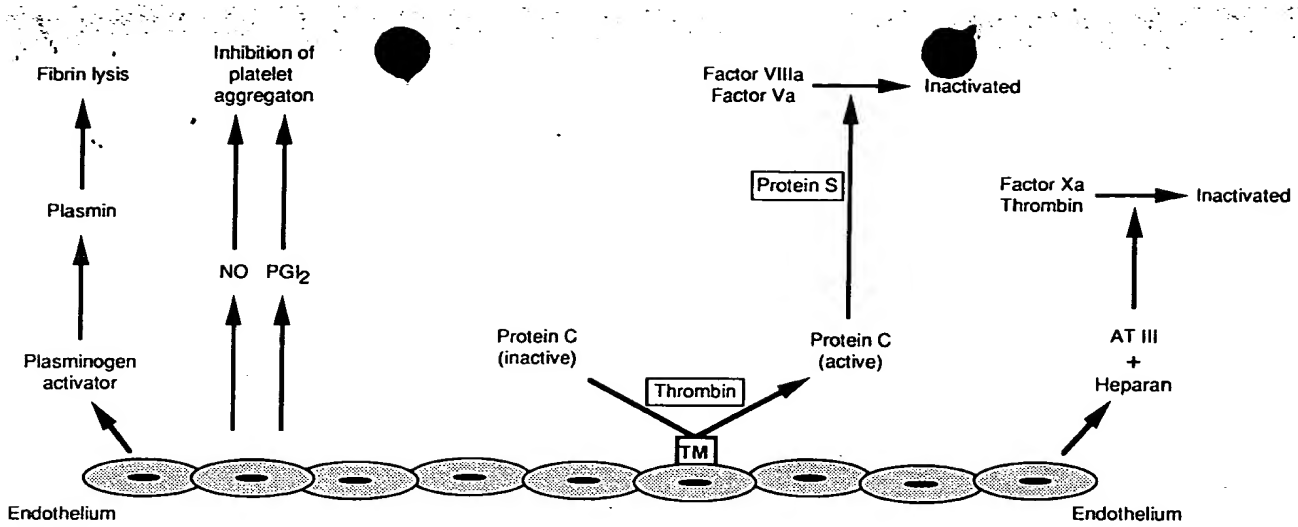

Fig. 1 *Thromboresistant properties of quiescent or undamaged endothelium. Endothelial cells produce heparan, thrombomodulin (TM), prostacyclin (PGI_2), nitric oxide (NO), or endothelial-derived relaxing factor and plasminogen activator, all of which prevent thrombosis. Protein C is converted to activated protein C by binding to TM in the presence of thrombin. Protein S serves as a cofactor in the deactivation of factors VIIIa and Va by activated protein C.*

AT III = antithrombin III

and extrinsic pathways as shown in Figure 2. After the binding of tissue factor to its enzyme, factor VIIa, initiation of the procoagulant stimulus results in the activation of 2 substrates, factor IX and factor X.[4] Tissue factor is produced constitutively by interstitial cells, but not by cells in contact with blood. However, increased expression of tissue factor by endothelial cells and macrophages has been demonstrated after treatment with a variety of agonists in vitro.[5] Particularly pertinent to the progression of atherosclerosis and thrombosis is the ability of TNF, IL 1, and VPF/VEGF to up-regulate the expression of tissue factor and their association with coagulopathies in vivo, because these 3 factors are thought

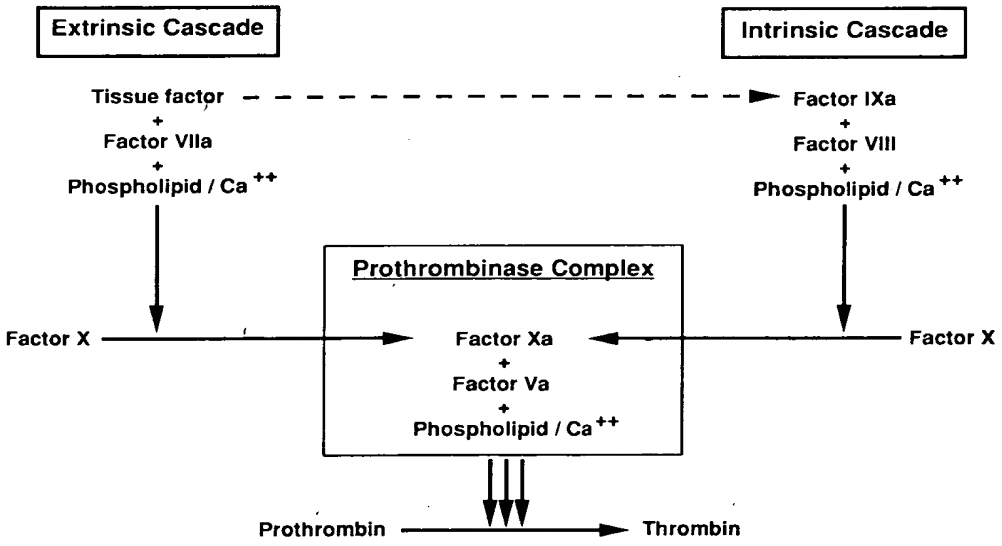

Fig. 2 *Formation of thrombin by intrinsic and extrinsic coagulation cascades that convert prothrombin to thrombin via the final common pathway, which involves the conversion of factor X to Xa. Stimulation of endothelial cells by immunomodulators such as tissue necrosis factor causes an up-regulation of tissue factor, which can initiate the extrinsic system through factor X activation and the intrinsic system via factor IX. The ability of factor IXa/factor VIIIa to activate factor X directly provides a mechanism for thrombin (factor IIa) generation when tissue factor concentrations are low. Generation of thrombin results in fibrin formation (not shown).*

to be produced within the microenvironment of the developing plaque and may contribute to thrombus formation.[1]

The final common pathway of thrombin formation is activated when factor Xa is complexed with its cofactor Va to form the prothrombinase complex, which converts prothrombin to thrombin (Fig. 2). The activated factor IX (factor IXa), complexed with its cofactor VIIIa, can also cleave factor X and contribute to the activation of the final common pathway. Recently, the importance of the feedback loop for factor IX activation has been proposed;[5,6] thrombin that has been generated via tissue factor–dependent mechanisms feeds back into the intrinsic pathway at the level of factor XI. This model suggests a mechanism for continued factor X activation during inhibition of the tissue factor pathway, which may block the tissue factor–mediated activation of factors IX and X.

Factor IXa and factor Xa are subject to regulation by the protein C–protein S pathway (Fig. 1). This mechanism is initiated by thrombin-mediated activation of protein C, which in turn deactivates factors Va and VIIIa. The 1st of these steps requires the cofactor thrombomodulin and the 2nd requires protein S, both of which are produced by and bound to the endothelial cell.[7,8] Stimulation of endothelial cell monolayers with TNF results not only in increased expression of tissue factor but also in decreased expression of thrombomodulin.[9] Exposure of the endothelium to TNF leads to augmentation of the synthesis of plasminogen activator inhibitor,[10] which pushes the balance to a procoagulant state with reduced fibrinolytic activity.

Tumor Necrosis Factor

Clearly, the contrast between intravascular thrombosis, on one hand, and the bleeding associated with disruption of hemostasis, on the other—both relatively rare under normal physiologic conditions—suggests a site of exquisite control. An in vivo model that was developed to study mechanisms of intravascular thrombin formation and fibrin deposition[11] used low-dose infusion of TNF in mice bearing the 3-methylcholanthrene A (Meth A)–induced fibrosarcoma. Within 30 minutes after TNF infusion, fibrin was detected on the tumor vasculature together with a marked increase in vascular permeability. This vascular collapse is thought to contribute to the in vivo sensitivity of TNF. The higher TNF sensitivity of the tumor endothelium compared with that of normal vessels suggested that endothelial cells within the tumor microenvironment may be altered.[11] Recently, 3 factors produced by Meth A cells in vitro have been identified: endothelial cell macrophage activating polypeptides I and II[12-14] and the endothelial cell mitogen, VPF/VEGF. These factors induce the expression of tissue factor on macrophages and act synergistically with TNF on endothelial cells to produce tissue factor. In vitro studies also showed the induction of leukocyte chemotaxis, and studies in vivo, an inflammation-producing effect.[14]

The TNF responsiveness of tumor vasculature can also be used as a model for cytokine-induced changes; in the presence of constitutively produced tumor factors the expression of tissue factor is most likely up-regulated. Further evidence for this up-regulation is provided by studies showing that fibrin deposition within the tumor can be measured within 30 minutes after TNF infusion.[11] Extrapolating from the available in vitro data for fibrin formation, such results suggest that this rate of fibrin formation is too rapid to be explained entirely by de novo synthesis of tissue factor. When endothelial cells were examined, only low levels of tissue factor activity were detected on intact monolayers with or without TNF stimulation. Whereas an approximately 3-fold increase in tissue factor activity was observed after treatment of intact monolayers of endothelial cells with TNF or IL 1,[15,16] a 10- to 20-fold increase was evident after monolayers were lightly permeabilized or removed in such a way as to expose the underlying extracellular matrix.[15] Such dramatic increases in tissue factor activity have also been shown using matrix isolated from TNF-treated endothelial monolayers under flow conditions.[17,18]

Tissue Factor

Tissue factor has 2 substrates, factor IX and factor X, and cellular binding sites have been described for both.[19-21] Specific binding of factor IX/IXa to endothelial cells and platelets has been demonstrated in vitro. The vitamin K–dependent γ-carboxyglutamic acid domain is essential to this interaction.[22-26] The rapid clearance of intravenously infused factor IX with its absorption onto the vessel wall suggests a high number of factor IX binding sites in vivo.[27] While factor IX/IXa binds to endothelial cells equally well (kDa = 2 nM), the affinity for factor IXa is greatly increased in the presence of factors X and VIIIa.[28] Thus, endothelial cells provide a site for the assembly of a factor X–activating complex.

The involvement of factor IXa in cytokine-mediated intravascular coagulation was suggested by the ability of active site-blocked factor IXa (factor IXai) to inhibit fibrin deposition in tumor microvessels when infused with TNF.[17] To further examine the relative importance of factors IX and X in intravascular coagulation, we used the inhibitor Glu-Gly-Arg–chlormethyl ketone, which binds irreversibly to the active site of factors IXa and Xa (factor IXai and factor Xai). These active site-blocked factors have no enzymatic activity but compete with the active enzyme for receptor, cofactor, and substrate binding.

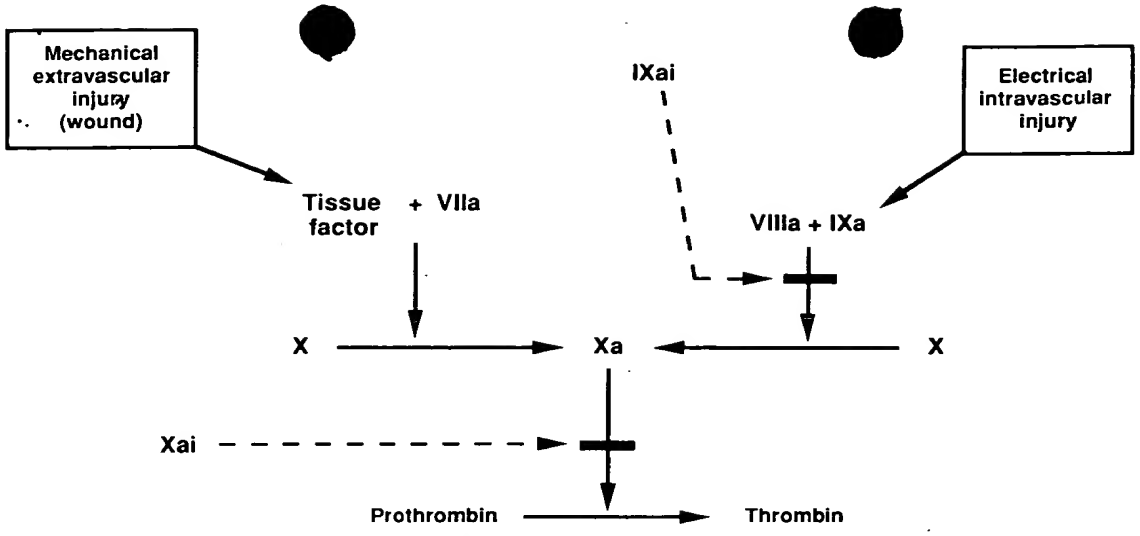

Fig. 3 *Depiction of the procoagulant mechanisms activated by mechanical extravascular damage due to subcutaneous wound or electrically induced intravascular injury. Inhibition is shown by broken lines. Active site-blocked Xa (Xai) inhibits the final common pathway and prevents both intra- and extravascular thromboses. In contrast, active site-blocked IXa (IXai) does not prolong extravascular bleeding and appears to be more important at the intravascular level where tissue factor concentrations are low.*

Active site-blocked factors IXa and Xa were compared for their ability to inhibit intra- and extravascular coagulation in dogs.[29] Both active site-blocked factors IXa and Xa caused a dose-dependent inhibition of intravascular thrombosis, initiated in the coronary arteries, indicating the importance of factor IXa in the intravascular activation of the final common pathway[29] (Fig. 3). However, when extravascular coagulation was measured in conjunction with blood loss from a standardized abdominal incision, infusion of factor IXai at concentrations sufficient to inhibit intravenous coagulation did not produce bleeding significantly different from that in control animals. In view of the role of factor Xa in the formation of an active prothrombinase complex and the consequent activation of the final common pathway, it was not surprising that factor Xai inhibited both extravascular hemostasis and intravascular thrombosis.[30] Previous in vivo and in vitro studies have suggested that factor X activation by the tissue factor–VIIa complex is favored where tissue factor concentrations are high (for example, in extravascular spaces such as the skin).[31,32] However, at low tissue factor concentrations, activation of factor IX is enhanced. Taken together with our findings, this evidence lends support to the view that factor IX/IXa serves an important function in intravascular thrombosis, which is separate from extravascular hemostasis (Fig. 3).

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